Layer guided-acoustic plate mode biosensors for monitoring MHC–peptide interactions†

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The transduction signals from the immobilisation of a class I heavy chain, HLA-A2, on a layer guided acoustic plate mode device, followed by binding of β_2-microglobulin and subsequent selective binding of a target peptide are reported.

Two key areas of investigation in immunology are Major Histocompatibility Complex (MHC)–peptide interactions and the construction and use of artificial systems for antigen presentation to T-lymphocytes (T cells). The MHC codes for a family of proteins that play a critical role in immune responses to cellular proteins and proteins derived from foreign organisms. MHC molecules bind peptide fragments derived from naturally processed proteins and display them on the surface of cells for recognition by T cells. In man there are 2 main classes of MHC: (HLA) class I antigens expressed on the surface of all nucleated cells and class II antigens which have a restricted expression profile and are found mainly on cells capable of antigen presentation. When expressing immunogenic peptides, class I and class II MHC molecules stimulate cytotoxic T-cell and T-helper cell responses respectively. HLA class I antigens are comprised of a HLA heavy chain and β_2m which is non-covalently linked. Peptides associated with this complex are then presented for recognition by the T-cell receptor expressed by T-lymphocytes and subsequently promote antigen-specific T-cell immune responses.

Potentially the changes in conformation as the HLA heavy chain refolds with its attendant partners may be detectable by a shift in the response of a surface sensitive resonant device. In particular, our interest is whether an acoustic wave response can provide both a quantification of the amount of peptide binding to the HLA and an estimate of the time for it to occur. An acoustic wave sensor suitable for this purpose operates in a liquid with high sensitivity to binding. This suggests a Love wave device, which uses a shear horizontal mode of oscillation with a guiding layer to obtain maximum sensitivity.[1] However, such a device has the guiding layer on the same face of the substrate as the inter-digital transducers (IDT) used for the generation and detection of the wave and both of these are also on the substrate face used for the binding specific surface chemistry. An alternative is to use a shear horizontal acoustic plate mode (SH-APM), which may be excited and detected by IDTs fabricated on the opposite face of the substrate to that used for sensing,[2,3] but this exhibits a lower sensitivity than Love waves.[4] Recently, McHale *et al.*[5,6] suggested from theoretical considerations that a guiding layer could be used on one substrate face of a SH-APM device to create a layer guided acoustic plate mode (LG-APM) and so obtain a sensitivity approaching that of a Love wave. Recently, Evans *et al.*[7] demonstrated experimentally that lithium tantalate substrates could be used for a LG-APM, thus creating the possibility of using opposing substrate faces to separate the layer providing enhanced sensitivity from that for the binding specific surface chemistry.

SH-APM devices were fabricated on 36° rotated Y-cut X propagating lithium tantalate substrates of thickness 540 μm with the underside of the wafers polished. The IDTs were a double–double finger design with 100 fingers, finger width and spacing of 20 μm and an aperture of 3000 μm; the distance from centre-to-centre of IDTs was 12 mm and the fingers consisted of 40 nm of titanium followed by 200 nm of gold. The fundamental frequency of the device was 26.3 MHz and a series of plate mode resonances were observed at 27.8, 29.5, 32.0, 36.2, 39.8, 43.6 and 47.6 MHz. Guiding layers of S1813 photoresist were spun onto either the upper-side or the under-side of the substrates at 1000 rpm for 5 seconds and then 4000 rpm for a further 30 seconds; the layers were then cross-linked by heating for 1 hour at 105 °C. Sensitivity measurements were made by depositing thin layers of gold in the form of a 4 mm wide stripe between the IDTs on to the guiding layer and monitoring the spectra of the devices using a network analyser; the resonant frequency was determined as the minimum in insertion loss for the different peaks. These measurements were used to choose a guiding layer thickness for optimum sensitivity in the biosensing experiments, which used a flow cell with a path length of 4 mm. Whilst the upper-side of the acoustic wave device could be used as the sensing side, in these experiments we used the under-side to apply a photoresist guiding layer and the upper-side, containing the IDTs, for a separate sensing layer of polystyrene on to which the MHC–peptide immobilisation was performed.

The immobilisation method was based on the strong non-covalent avidin–biotin interaction[8] and involved the addition of a photoactivatable biotin residue onto a polystyrene surface; polystyrene has previously been shown to be robust in the presence of buffer liquids.[9] The polystyrene layer of thickness 1.87 μm was spin coated at 860 rpm in a 10 mg ml^{-1} solution in toluene. Following deposition the layer was cleaned by exposure to ozone for 30 minutes. Once the device had been coated with polystyrene, the photobiotin acetate (Sigma Chemicals) in a solution of 10 mg ml^{-1}

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ethanol–water 20 : 80% was applied and left overnight under nitrogen after which it was fixed in place by exposure to UV; the surface was then able to bind the streptavidin (molecular weight of 55–60 kDa). The LG-APM device was then placed into a flow cell into which was passed phosphate buffered saline (PBS) 20 mM drawn across by a peristaltic pump at 0.4 ml min^{-1} and the acoustic phase was monitored until a stable signal was achieved. Solutions were temperature equilibrated prior to being introduced to the device surface *via* a slow flow system with a 10 cm path length.

Initial studies looked at introducing streptavidin at 0.1 mg ml^{-1} and following this with biotinylated HLA-A2 heavy chain[10] at 0.3 mg ml^{-1}; the molecular weight of the HLA-A2 is 45 kDa. However, this did not produce reliable results and an approximately 2-to-1 cocktail of streptavidin (0.15 mg ml^{-1}, Sigma Chemicals) and biotinylated HLA-A2 heavy chain (0.3 mg ml^{-1}) was introduced. The resulting sensor surface is shown schematically in Fig. 1(a); since streptavidin has four biotin binding sites, this procedure will produce a mixture of surface bound streptavidin with between one and four binding sites linking to the sensor surface and the remaining sites binding with the HLA-A2 heavy chain. At this stage the HLA-A2 heavy chain is unfolded and the α_2 and α_3 constituent domains remain flexible. The binding of each component (biotin and then streptavidin) *via* this approach was confirmed by fluorescence microscopy. The next stage was to challenge the sensor with 0.3 mg ml^{-1} of β_2m[11] to initiate partial refolding of the heavy chain thus initiating the formation of the peptide binding cleft; β_2m has a molecular weight of 11.5 kDa. At this stage the β_2m and HLA-A2 molecule is still relatively unstable and can disassociate easily; a schematic of the sensor in this configuration is shown in Fig. 1(b). This system of HLA-A2 heavy chain and β_2m was then challenged with the CMV derived pp65 NLVPMVATV–peptide (molecular weight of 0.95 kDa), which is known to bind strongly, and also with a β-galactosidase peptide epitope TPHPARIGL that binds only weakly with the MHC (Fig. 1(b)). After peptide binding has occurred the molecule attains its final conformation with all components more rigidly bound. Fig. 1(b) shows a schematic of the complete sensor including the transduction components of the sensor comprising of the photoresist guiding layer on the lower substrate face of the lithium tantalate (to increase sensitivity) and the IDT's used to launch and receive the acoustic wave; The biotin linked polystyrene–streptavidin–HLA-A2–β_2m structure provides the peptide specific binding surface chemistry.

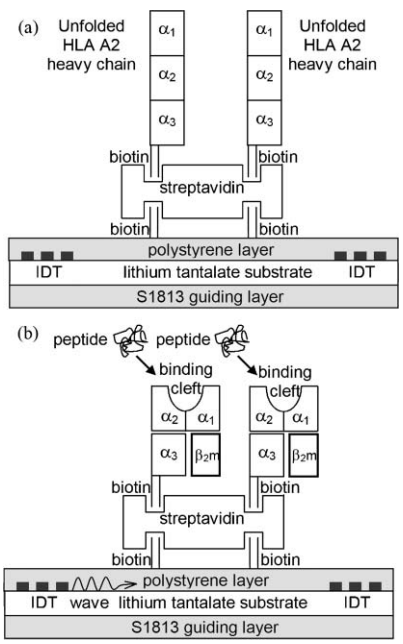

Fig. 1 Schematic of sensor: (a) substrate with streptavidin–biotin binding to unfolded HLA-A2 chain and with biotin binding to polystyrene layer to link to the acoustic wave sensor; (b) binding of β_2m causing partial refolding with subsequent binding of peptide sensed by the acoustic wave.

A first set of experiments was used to assess the relative mass sensitivities provided by guiding layers on opposing faces of the substrate and, hence, to select an operating guiding layer thickness. Fig. 2(a) shows the change in resonant frequency of a 47.6 MHz plate mode as gold is deposited on to a guiding layer of thickness 14 μm. In this figure, the diamonds show the effect of the gold deposited on a guiding layer on the upper face containing the IDT and the squares show the effect of the gold deposited on a guiding layer on the opposite (lower) face; in both cases only one face of the substrate was coated. The gradients from Fig. 2(a) are 6.5 kHz nm^{-1} (upper face guiding layer) and 6.2 kHz nm^{-1} (lower face guiding layer) for an effective sensing area of 0.12 cm^2 suggesting that similar sensitivity is achieved for a guiding layer on either face; this corresponds to frequency sensitivities of 336 Hz (ng $mm^{-2})^{-1}$ and 321 Hz (ng $mm^{-2})^{-1}$. To systematically determine the optimum guiding layer thickness, this procedure

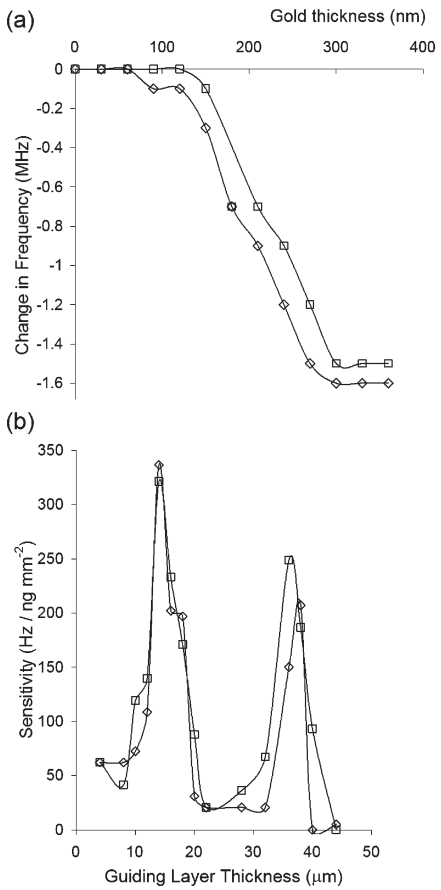

Fig. 2 (a) Change in resonant frequency of the 47.6 MHz mode as a function of thickness of gold deposited on the guiding layer. The diamonds show data for the guiding layer on the IDT face and the squares for the guiding layer on the opposite face. (b) Sensitivity calculated from the gradients in Fig. 2(a) as a function of guiding layer thickness.

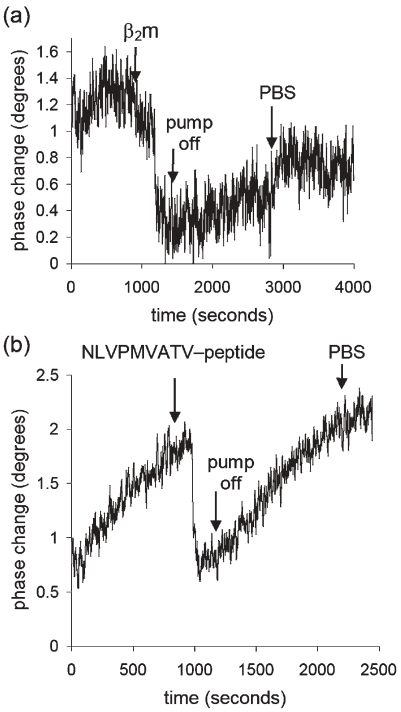

Fig. 3 (a) Change in phase as a result of the addition of β_2m at 870 seconds with the pump switched off at 1430 seconds and restarted with PBS at 2873 seconds. (b) Change in phase as a result of the addition of NLVPMVATV–peptide at 830 seconds with the pump switched off at 1156 seconds and restarted with PBS at 2230 seconds.

was repeated for guiding layer thicknesses up to 44 μm; Fig. 2(b) summarizes the resulting dependence of sensitivity on guiding layer thickness. These values are comparable with traditional APM sensors[3] operating at several times the frequency used for our LG-APM, thus confirming the gain in sensitivity from the use of a guiding layer. From these data a guiding layer thickness of 14 μm, corresponding to the peak in sensitivity for the first guided mode, was chosen for the subsequent bio-sensing experiments. Based on the measured frequency sensitivity of 321 Hz (ng mm^{-2})$^{-1}$ and using an estimate of the wavelength of the 47.6 MHz plate mode of 100 μm, the phase sensitivity of the system is ~0.1 degrees per ng mm^{-2}.

The bio-sensing experimental sequence was initiated by the introduction of the streptavidin and HLA-A2 heavy chain together to the sensing surface, then pausing the pump for a period of approximately 30 minutes followed by restarting the flow system with buffer. Subsequently, the β_2m was introduced and this produced an approximately 1° fall in the acoustic phase (Fig. 3(a) at 830 s). At 1430 s the pump was switched off to allow the surface to be in contact with the β_2m and the pump was then restarted with buffer at 2873 seconds. The β_2m binds to the HLA-A2 heavy chain causing partial refolding of the A2 (Fig. 1(b)). Given the relative size of the β_2m, which is a small protein of molecular weight 11.5 kDa, we believe the ability of the sensor to detect the change in phase by a mass loading response is marginal. Assuming the streptavidin forms a rigidly packed monolayer with typical molecular diameter of 84 Å[12] the surface mass density can be estimated as $\Delta m_s \sim 1.8$ ng mm^{-2}. Using an average of two HLA and hence two β_2m per streptavidin then suggests a surface mass density for the β_2m of $\Delta m_{\beta 2m} \sim 0.7$ ng mm^{-2} and this implies an acoustic wave phase change ~ 0.07° based on the sensitivity determined from Fig. 1(b); this is an order of magnitude less than observed in Fig. 3(a). The conformational changes taking place as the HLA-A2 heavy chain partially refolds (*i.e.* the transition shown schematically from Fig. 1(a) to Fig. 1(b)) may therefore be the cause of the change in phase.

The subsequent change of phase as the NLVPMVATV–peptide is introduced to this system is shown in Fig. 3(b). Although thermal drift on the data is present, the sharp change upon introduction of the peptide was repeatable. The molecular weight of the peptide is 0.95 kDa, which is around 8% of the molecular weight of the β_2m, but nevertheless we observe a similar size phase change as occurred upon the introduction of the β_2m and this is strongly indicative of a non-mass loading response. Since it is expected that the MHC should respond to this particular peptide entering the binding cleft by completely folding and the components becoming more rigidly bound, we believe this conformational change could be the cause of the measured phase change. To verify whether the binding was peptide specific, the experimental procedure was repeated with the last stage using a TPH epitope that binds only weakly with the MHC and no phase shift was observed in this case (exemplar data provided in the electronic supplementary information†).

In this study we have demonstrated that guiding layers on an acoustic plate mode device can be used to enhance the sensitivity without losing the advantages of the traditional APM. The application of the layer guided APM device as a biosensor has been demonstrated by monitoring the formation of an HLA molecule from an immobilised HLA-A2 heavy chain and the addition of β_2m to create a partially refolded product. The subsequent binding of a peptide with a known affinity has then been observed. The signal response has been interpreted to be a consequence of a conformational change because a simple mass attachment would be expected to provide an acoustic response lower than the detection threshold. A similar experiment with a weakly binding peptide showed no detectable change. We have therefore demonstrated that LG-APMs provide potential for real time monitoring of MHC–peptide interactions. The use of a higher fundamental frequency LG-APM to increase the sensitivity should allow this approach to become a tool for assessing protein–protein/protein–peptide binding.

Notes and references

1 E. Gizeli, A. C. Stevenson, N. J. Goddard and C. R. Lowe, *IEEE Trans. Ultrason. Ferroelec. Freq. Control*, 1992, **39**, 657.
2 S. J. Martin, A. J. Ricco, T. M. Niemczyk and G. C. Frye, *Sens. Actuators*, 1989, **20**, 253.
3 F. Josse, D. Dahint, J. Schumacher, M. Grunze and J. C. Andle, *Sens. Actuators, A*, 1996, **53**, 243.
4 J. D. N. Cheeke and Z. Wang, *Sens. Actuators, B*, 1999, **59**, 146.
5 G. McHale, M. I. Newton and F. Martin, *J. Appl. Phys.*, 2002, **91**, 9701.
6 G. McHale, M. I. Newton and F. Martin, *J. Appl. Phys.*, 2002, **91**, 5735.
7 C. R. Evans, S. M. Stanley, C. J. Percival, G. McHale and M. I. Newton, *Sens. Actuators, A*, 2006, DOI: 10.1016/j.sna.2006.01.030.
8 S. Xiaodi, *Biochem. Biophys. Res. Commun.*, 2002, **290**, 962.
9 D. W. Branch and S. M. Brozik, *Biosens. Bioelectron.*, 2004, **19**, 849.
10 *Lymphocytes, a practical approach*, ed. G. G. B. Klaus, S. L. Rowland-Jones and A. J. McMichael, 2nd edn, Oxford University Press, Oxford, 1999.
11 R. C. Hillig, P. G. Coulie, V. Stroobant, W. Saenger, A. Ziegler and M. Hulsmeyer, *J. Mol. Biol.*, 2001, **310**, 1167.
12 S. A. Darst, M. Ahlers, P. H. Meller, E. W. Kubalek, R. Blankenburg, H. O. Ribi, H. Ringsdorf and R. D. Kornberg, *Biophys. J.*, 1991, **59**, 387.

